REMARKS

Entry of this Amendment is proper under 37 C.F.R. § 1.116, because the Amendment places the application in condition for allowance for the reasons discussed herein; does not raise any new issue requiring further search and/or consideration, because the amendments amplify issues previously discussed throughout prosecution (*i.e.*, claim 1 as amended introduces the elements from claims 7 and 10-11); does not present any additional claims; relates to matters of form rather than substance, because the added language was already present in the claims, and places the application in better form for an appeal should an appeal be necessary. The Amendment is necessary and was not earlier presented, because it is made in response to arguments raised in the final rejection. Entry of the Amendment, reexamination, and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.116, are thus respectfully requested.

The Office is respectfully requested to contact the undersigned representative for further discussion in view of the presently submitted amendments and remarks. Applicants further request an interview with the Examiner Richard A. Schnizer, Ph.D. to clarify any issues of the above-identification application.

1. Status of the Claims

Claims 1 and 3-11 stand pending. Claims 1 and 3-11 stand rejected. Claims 2 and 12-15 stand previously canceled.

After entry of the above amendments, claims 7-11 stand canceled. Applicants amend claims 1 and 3-6 to more precisely recite the claimed subject matter. Support for the amendments can be found at least, for example, from the claims previously presented. Accordingly, no prohibited new matter is introduced by entry of the present amendment.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicants reserve the right to file a continuation or divisional application on any subject matter canceled by way of amendment.

2. Priority

Applicants note that the Office has asserted that the effective filing date of the claims is no earlier than the filing date of PCT/JP2005/05786, *i.e.*, March 26, 2005, because no English language translation was provided for the Japanese priority application, JP 2004-107512, which was filed March 31, 2004.

Applicants attach hereto a verified English translation of JP 2004-107512. By perfecting priority with this translation, Applicants claim benefit of the filing date of March 31, 2004. *See* M.P.E.P. § 706.02(b).

3. Rejection of the Claims Under 35 U.S.C. § 103(a)

3.1. Rejection of claims 1, 3-4, 6-9, and 11

The Office maintains the rejection of claims 1, 3-4, 6-9, and 11 over the following three references:

1) **Certik** M. *et al.*, *Desaturase-Defective Fungal Mutants: Useful Tools for the Regulation and Overproduction of Polyunsaturated Fatty Acids*, 16 TRENDS BIOTECHNOL. 500 (1998) [hereinafter "Certik"];

2) **Ueda** R., *RNAi: A New Technology in the Post-Genomic Sequencing Era*, 15 J. NEUROGENETICS 193 (2001) [hereinafter "Ueda"]; and

3) **Mackenzie** D.A. *et al.*, *Isolation and Use of a Homologous Histone H4 Promoter and a Ribosomal DNA region in a Transformation Vector for the Oil-producing Fungus* Mortierella alpina, 66 APPL. ENVIRON. MICROBIOL. 4655 (2000) [hereinafter "Mackenzie"].

Certik relied upon for allegedly teaching that desaturase-defective *Mortierella alpina* mutants are useful to produce polyunsaturated fatty acids (PUFAs). Page 3, Office Action. Ueda allegedly teaches RNAi as a means of selectively inhibiting expression of genes that are conserved across plants, animals, and fungi. *Id.*, pages 3-4. Mackenzie allegedly teaches delivering genetic material to achieve stable expression in *Mortierella*. *Id.*, page 4. The Office alleges that a skilled artisan, in view of Certik, Ueda, and Mackenzie, would have been motivated to employ RNAi to inhibit the activity of *M. alpina* desaturases. *Id.* Additionally, the

Office asserts that one would have had a reasonable expectation of success in view of (1) Ueda's teaching that RNA interference has been known to function in fungi, and (2) Mackenzie's teaching that vectors and techniques are available for establishing stable expression of heterologous genes in *M. alpine*. *Id.*

In prior Amendments / Response, Applicants argue that none of the references teaches a co-suppression method as recited. The Office discounts the arguments, alleging that (1) the RNAi method and the co-suppression method are recited as alternatives; and (2) the RNAi method is a species of the genus of phenomena recognized as co-suppression. Page 8, Office Action.

Applicants further argue that there was no reasonable expectation of success in combining the cited references to suppress a specific gene in *Mortierella* through either an RNAi method or a co-suppression method, because there has been no report as to the effectiveness of the RNAi method in lipid producing *Mortierella* at the priority date of the present application. The Office asserts that the argument is unpersuasive and unsupported, alleging that a reasonable expectation of success required for *prima facie* obviousness differs from the certainty of success. The Office further alleges that the existence of conserved RNAi pathway across phylogenetic kingdoms, as taught by Ueda, would have provided a reasonable expectation that (1) the fungal species *M. alpine* would have a functional RNAi pathway, and (2) one could carry out RNAi in this species. Pages 9-10, Office Action.

Applicants had also argued in the prior Amendments / Response that the recited methods are capable of fine-tuning the level of suppression, which amounts to unexpected results. The Office discounts the argument, referring to Ueda's teaching that the RNAi method is known to result in incomplete inhibition. Pages 10-11, Office Action.

Applicants traverse. A finding of obviousness under 35 U.S.C. § 103 requires a determination of the scope and content of the prior art, the differences between the invention and the prior art, the level or ordinary skill in the art, and whether the differences are such that the claimed subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. *Graham v. John Deere Co.*, 383 U.S. 1, 148 U.S.P.Q. 459

(1966); *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 82 U.S.P.Q.2d 1385 (2007). When determining whether a claim is obvious, the Office must make "a searching comparison of the claimed invention–including all its limitations–with the teaching of the prior art." *In re Ochiai*, 71 F.3d 1565, 1572, 37 U.S.P.Q.2d 1127, 1133 (Fed. Cir. 1995). Further, "obviousness requires a suggestion of ***all*** limitations in a claim." *CFMT, Inc. v. Yieldup Int'l Corp.*, 349 F.3d 1333, 1342, 68 U.S.P.Q.2d 1940, 1947 (Fed. Cir. 2003) (emphasis added). Once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful. *In re Vaeck*, 947 F.2d 488, 493, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991); *Examination Guidelines for Determining Obviousness under 35 U.S.C. 103 in View of the Supreme Court Decision in* KSR International Co. v. Teleflex Inc., 72 Fed. Reg. 57,528. This includes assessing the references for what they teach as a whole and not particulate dissection of a reference for piecemeal hindsight determinations of obviousness through the benefit of Applicants' own specification as guidance.

Contrary to the Office's position, the information at the time of filing would not have provided the skilled artisan with a reasonable expectation of success that the claimed breeding method would have worked. (1) It was not possible at the time to predict at what level the precursors would accumulate. Accordingly, it would not have been possible to predict the fatty acid accumulation given the co-suppression. (2) In the case of the Δ6 fatty acid desaturase, there are two genes in *Mortierella*. It was unpredictable at the time that both genes could be co-suppressed using a construct based on the sequence of only one of the two genes. (3) For all the genes, it would not have been reasonably been expected that suppression of a lipid metabolism gene in *Mortierella* would have worked by using an RNAi method or by using a co-suppression method. Applicants provide the following evidence showing that a general teaching that RNAi pathways exist in fungi does not provide a reasonable expectation that:

1) the RNAi pathway exists in a particular fungal species, and

2) a skilled artisan could carry out RNAi-mediated suppression in a particular fungal species.

In support of this argument, Applicants herewith direct the Office to the post-filing non-patent

references of (1) **Proudfoot** N. and Gullerova M., *Gene Silencing CUTs Both Ways*, 131 CELL 649 (2007) (Annex I) [hereinafter "Proudfoot"] ; and (2) **Nakayashiki** H. and Nguyen Q.B., *RNA Interference: Roles in Fungal Biology*, 11 CURR. OPIN. MICROBIOL. 494 (2008) (Annex II) [hereinafter "Nakayashiki"]. Proudfoot states:

> **In the fission yeast *Saccharomyces pombe*, gene silencing has been shown unexpectedly to involve the RNA interference (RNAi) pathway** and in particular the RNase III enzyme Dicer... **However, the budding yeast *S. cerevisiae* seems bereft of RNAi-mediated gene regulation**, as it lacks Dicer, Argonaute proteins, and RNA-dependent RNA polymerase. (Emphasis added).

Page 649, bottom parts of col. 1 and col. 3, Proudfoot. Similarly, Nakayashiki states:

> **In some fungal species, such as *S. cerevisiae* and *Ustilago maydis*, the entire RNA silencing machinery appears to be lost, indicating that RNA silencing pathways may be dispensable for fundamental metabolism and development in fungi.** Interestingly, in *Ustilago hordei*, a close relative to *U. maydis*, RNA silencing has been demonstrated. Therefore, **the loss of the RNA silencing machinery seems to sporadically occur in the fungi kingdom**, as previously shown in the protest *Trypanosomes*. In support of this, *A. nidulans* appears to be 'losing' one of the two copies of dicer and argonaute genes while, intriguingly, its close relatives *Aspergillus oryzae* and *Aspergillus flavus* have likely gained extra copy of dicer and argonaute genes, possibly by gene duplication. (Emphasis added).

Page 498, bottom portion of the left col. and upper portion of the right col. Proudfoot and Nakayashiki combined teach that RNAi machinery cannot be expected because it may be "sporadically" lost in certain fungal species, such as *Saccharomyces cerevisiae* and *Ustilago maydis*. This loss can occur even while the machinery remains intact in close relatives, such as *S. pombe* and *U. maydis*, respectively.

Accordingly, a skilled artisan could not have had a reasonable expectation that the RNAi machinery existed in a particular fungal species, *i.e.*, *Mortierella*. It should be noted that these references that describe this issue were published after the filing of the instant application, further lending support that the skilled artisan would not have had the benefit of this information at the time. The cited references evince that no reasonable expectation of success that this putative combination could have successfully worked, *i.e.*, achieving suppression of a lipid metabolism gene in *Mortierella* through either an RNAi method or a co-suppression method. The amended claims are thus non-obvious over cited references. *See Vaeck*, 947 F.2d at 493, 20

U.S.P.Q.2d at 1442.

Claims 7-9 and 11 stand canceled upon entry of the present amendment, mooting this aspect rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

3.2. Rejection of claims 1 and 3-8

The Office maintains the rejection of claims 1 and 3-8 over **Takeno** S. *et al.*, *Establishment of an Overall Transformation System for an Oil-Producing Filamentous Fungus, Mortierella alpina* 1S-4, 65 APPL. MICROBIOL. BIOTECHNOL. 419 (2004) [hereinafter "**Takeno**"] as applied to claim 1 and further in view of Ueda and Mackenzie.

Applicants traverse. The basis for determining obvious is discussed in Section 3.1. *supra.*

Upon entry of the present amendment, each claim recites a method of breeding lipid producing *Mortierella* by suppressing expression of a lipid metabolism gene with an RNAi method or a co-suppression method. With the submission of a verified English translation of JP 2004-107512, Applicants have perfected priority to the instant Japanese application and the filing date of March 31, 2004. *See* Section 2. *supra.* As the perfected filing date antedates the on-line publication date of Takeno, May 12, 2004, Takeno is no longer available as a prior art reference. Ueda and Mackenzie, alone or combined, fail to teach suppressing expression of a lipid metabolism gene in *Mortierella*, as recited in each claim as argued in Section 3.1. *supra.* There is no *prima facie* case of obviousness, because the alleged prior art references fail to teach or suggest all claim elements. *CFMT*, 349 F.3d at 1342, 68 U.S.P.Q.2d at 1947. Additionally, claims 7-8 stands canceled upon entry of the present amendment, mooting the rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claims.

3.3. Rejection of claim 5

The Office maintains the rejection of claim 5 over Certik and Mackenzie as applied to claims 1, 3, 4, 6-9, and 11, and further in view of **White** T.C. *et al.*, U.S. Patent No. 6,939,704

[hereinafter "White"]. White allegedly teaches that filamentous fungi could be transfected by calcium chloride treatment, electroporation, or particle bombardment. Page 7, Office Action. The Office admits that the combination of Certik, Ueda, and Mackenzie fails to teach gene delivery by electroporation or particle bombardment. *Id.,* at page 6. The Office apparently applies White to cure this defect.

Applicants traverse to the extent that the rejection is maintained as to amended claim 5. The legal basis for obviousness is discussed in Section 3.1, *supra.*

White purportedly teaches methods of transafection for filamentous fungi. However, White does not teach suppressing expression of a lipid metabolism gene in *Mortierella* through an RNAi method or a co-suppression method, which is presently recited in each claim. As discussed in Section 3.1, *supra.* Certik, Ueda, and Mackenzie alone and combined fail to provide a reasonable expectation of success to suppress the expression of a lipid metabolism gene in *Mortierella* through an RNAi method or a co-suppression method. The addition of White fails to cure the defects of Certik, Ueda, and Mackenzie.

Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

3.4. Rejection of claim 9-10

The Office maintains the rejection of claims 9-10 over Certik, Ueda, and Mackenzie as applied to claims 1, 3, 4, 6-9, and 11, and further in view of **Parker-Barnes** J.M. *et al.*, *Identification and Characterization of an Enzyme Involved in the Elongation of n-6 and n-3 Polyunsaturated Fatty Acids*, 97 PROC. NAT. ACAD. SCI. USA 8248 (2000) [hereinafter "Parker-Barnes"]. Parker-Barnes allegedly teaches a gene encoding a fatty acid elongase from *M. alpina.* Page 7, Office Action. The Office alleges that a skilled artisan, in view of Certik, Ueda, and Mackenzie, would have been motivated to utilize the RNAi method to suppress the elongase gene disclosed in Parker-Barnes. Page 8, Office Action.

Applicants traverse the rejection for the record. Applicants note that after entry of the above amendments, claims 9-10 stand canceled thereby mooting the rejection.

While Parker-Barnes purportedly teaches a *M. alpine* fatty acid elongase, Parker-Barnes

fails to teach suppressing expression of a lipid metabolism gene in *Mortierella* through an RNAi method or a co-suppression method, which is presently recited in each claim. As discussed in Section 3.1. *supra*, Certik, Ueda, and Mackenzie combined fail to provide a reasonable expectation of success to suppress the expression of a lipid metabolism gene in *Mortierella* through an RNAi method or a co-suppression method. Additionally, the references of Proudfoot and Nakayashiki further evince that there could have been no expectation of success that the claimed method would have worked.

Applicants respectfully request withdrawal of the rejection and allowance of the claims in light of the amendments to the claims and above listed arguments.

In view of the above, the Office is respectfully request to withdraw all obviousness rejections and allow claims 1 and 3-6.

CONCLUSION

Should the Examiner have any questions or comments regarding Applicants' amendments or response, please contact Applicants' undersigned representative at (202) 842-8821. Furthermore, please direct all correspondence to the below-listed address.

In the event that the Office believes that there are fees outstanding in the above-referenced matter and for purposes of maintaining pendency of the application, including Notice of Appeal, the Office is authorized to charge the outstanding fees to Deposit Account No. 50-0573. The Office is likewise authorized to credit any overpayment to the same Deposit Account Number.

Respectfully submitted,

DRINKER, BIDDLE & REATH LLP

Date: August 6, 2009

Mercedes K. Meyer, Ph.D., Esq.
Registration No. 44,939

CUSTOMER NO. 55694
DRINKER, BIDDLE & REATH LLP
1500 K Street, N.W., Suite 1100
Washington, D.C. 20005-1209
Tel: (202) 842-8800; Fax: (202) 842-8465

The results from this new work are important not only because of the possible therapeutic implications for Gaucher disease but also because they highlight the fundamental difference between transport to lysosomes mediated by LIMP-2 and by the mannose 6-phosphate receptor (Figure 1). Recognition of the mannose 6-phosphate carbohydrate modification on lysosomal hydrolases by the mannose 6-phosphate receptor occurs in the *trans*-Golgi network, whereas LIMP-2 recognition of βGC can take place in the ER and is apparently carbohydrate independent. Lysosomal hydrolases dissociate from the mannose 6-phosphate receptor in late endosomes as this receptor is not present in lysosomes, whereas LIMP-2 is present in lysosomes, suggesting that dissociation of βGC from LIMP-2 may occur in the lysosome itself. It is not clear whether LIMP-2 might mediate sorting of other proteins to the lysosome. It is also not clear whether LIMP-2 delivers newly synthesized proteins, endocytosed proteins, or both to lysosomes. However, the low levels of LIMP-2 found on the plasma membrane suggest that it may act primarily inside the cell.

These results provide yet another example of where the study of a lysosomal storage disease has provided insights into the fundamental mechanisms controlling protein sorting in cell biology. But what insights into Gaucher disease do these findings provide? One intriguing possibility raised by the authors is that some patients with Gaucher-like phenotypes might have mutations in LIMP-2 that might prevent proper sorting of βGC. Further studies of LIMP-2-deficient mice will certainly provide insights into this possibility. The current studies also open up new ways in which to understand the loss-of-function mutations in βGC found in patients with Gaucher disease in terms of their recognition and transport by LIMP-2. Furthermore, by identifying the machinery that governs the secretion of βGC, these studies may provide ways to improve production of βGC, which is used therapeutically to treat patients with Gaucher disease. But perhaps most importantly these studies highlight the enormous synergy between studies of genetic diseases and our understanding of cell biology.

REFERENCES

de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955). Biochem. J. *60*, 604–617.

Kornfeld, S. (1990). Biochem. Soc. Trans. *18*, 367–374.

Reczek, D., Schwake, M., Schroder, J., Hughes, H., Blanz, J., Jin, X., Brondyk, W., Van Patten, S., Edmunds, T., and Saftig, P. (2007). Cell, this issue.

Rijnboutt, S., Aerts, H.M.F.G., Geuze, H.J., Tager, J.M., and Strous, G.J. (1991). J. Biol. Chem. *266*, 4862–4868.

Gene Silencing CUTs Both Ways

Nick Proudfoot[1,*] and Monika Gullerova[1]
[1]Sir William Dunn School of Pathology, South Parks Rd., OX1 3RE, University of Oxford, Oxford, UK
*Correspondence: nicholas.proudfoot@path.ox.ac.uk
DOI 10.1016/j.cell.2007.11.003

There is extensive transcription throughout the eukaryotic genome resulting in both antisense transcripts from coding regions and cryptic unstable transcripts (CUTs) from intergenic regions. In this issue, Camblong et al. (2007) demonstrate in the budding yeast that antisense transcripts, if stabilized by exosome impairment, are able to mediate gene silencing via the recruitment of histone deacetylases.

Silencing is a critical feature of gene regulation in both unicellular and higher eukaryotes. In the fission yeast *Saccharomyces pombe*, gene silencing has been shown unexpectedly to involve the RNA interference (RNAi) pathway and in particular the RNase III enzyme Dicer. In higher eukaryotes, Dicer is responsible for cutting up double-stranded RNA (dsRNA) into either small-interfering RNAs (siRNAs) or microRNAs that then regulate mRNAs through their incorporation into the RISC complex. This RNA-primed complex targets specific mRNAs, either promoting their degradation or inhibiting their translation. Instead, in *S. pombe*, Dicer is associated with a chromatin-silencing complex (RITS) that is recruited to regions of heterochromatin caught in the act of low-level transcription from both template strands thereby generating dsRNA. Indeed, most other higher eukaryotes share this RNAi-induced gene-silencing pathway (Grewal and Jia, 2007). However, the budding yeast *S. cerevisiae* seems bereft of RNAi-mediated gene regulation, as it lacks Dicer, Argonaute proteins, and RNA-dependent RNA polymerase. Yet, gene silencing still occurs in *S. cerevisiae*. So how is it achieved?

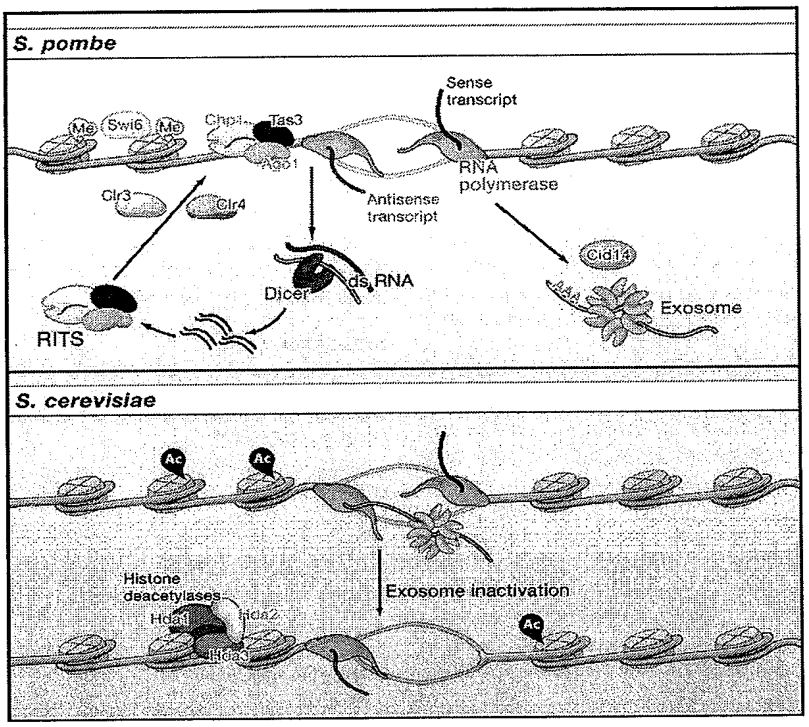


Figure 1. Parallel Mechanisms for Gene Silencing in Yeast
(Top) In *S. pombe*, heterochromatin is maintained through synthesis of double-stranded RNA (dsRNA) generated through low-level transcription from both DNA strands by RNA polymerase II with subsequent amplification by RNA-dependent RNA polymerase. Double-stranded RNA is degraded either by the exosome or Dicer. Dicer generates siRNAs that are incorporated into the RITS complex, which then targets homologous chromatin resulting in gene silencing by histone tail deacetylation and methylation followed by recruitment of Swi6. (Bottom) In *S. cerevisiae*, specific genes generate antisense transcription, which is normally suppressed by exosome-mediated degradation. However, if exosome activity is limited, antisense cryptic unstable transcripts (CUTs) accumulate. This causes recruitment of histone deacetylases to nearby chromatin, resulting in gene silencing. Swi6 is an Hp1 homolog; Clr3 and Hda1-3 are histone deacetylases; Clr4 is a histone methyltransferase; Cid14 is an exosome-activating polyA polymerase.

The study by Camblong et al. (2007) in this issue provides new exciting clues. They show that the stabilization of antisense RNAs in *S. cerevisiae* can lead to the recruitment of histone deacetylases to inhibit sense transcription (Figure 1).

In *S. cerevisiae*, transcriptional interference occurs if transcription across one gene fails to terminate. This results in read-through transcription, which causes downregulation of the next gene along the chromosome (Shearwin et al., 2005). If the downstream gene faces in the same direction (in tandem) then its promoter may be blocked by the read-through transcription (Greger et al., 2000). Such an interference process occurs naturally in *S. cerevisiae* for the *SER3* gene where an upstream promoter generates a cryptic unstable transcript (CUT) reading into *SER3* that then regulates the activity of the *SER3* gene by blocking transcription initiation (Martens et al., 2004). Alternatively, if the read-through transcript extends into a convergent downstream gene then polymerases may collide with each other causing an elongation block for both transcripts (Prescott and Proudfoot, 2002). This mechanism is adopted by the *IME4* gene, which controls the onset of meiosis in diploid cells. Here, the gene appears to exist as two wholly overlapping transcription units. When the antisense gene is active, as occurs in haploid cells, then *IME4* transcripts are switched off. However in diploid cells of mixed a/α mating type, an a/α repressor protein is synthesized that blocks the antisense promoter. This allows *IME4* transcription to take off (Hongay et al., 2006) and consequently allows the a/α diploid cell to proceed to meiotic division. Exactly how antisense transcription blocks sense transcription is unresolved.

The efforts of Camblong et al. provide new insight into how antisense transcription can block gene expression in *S. cerevisiae*. Like many important discoveries this story begins with a serendipitous finding. It was noticed that transcription from the *PHO84* gene progressively decreases if yeast colonies are artificially aged on minimum medium plates or in cultures left in the fridge for a few weeks. Other genes including those neighboring *PHO84* are unaffected by this ill treatment, suggesting that *PHO84* possesses a specific gene-silencing mechanism that is promoted under stress conditions. Indeed, a low-level antisense CUT is found to accumulate at the expense of *PHO84* mRNA, and (as with other CUTs) knockdown of the RNA degradation apparatus, in particular nuclear exosome components (Wyers et al., 2005), increases levels of the antisense CUT to further impair *PHO84* sense transcription. The authors then show that binding of the exosome (Rrp6) to chromatin associated with the antisense CUTs is diminished in aged cells and this presumably is the cause of the age-associated effect.

So how does the antisense *PHO84* transcript silence the gene without using RNAi's trickery? The authors resolve this question by showing that histone deacetylases (Hda1-3) are specifically recruited to antisense-repressed *PHO84* resulting in inactivation of the *PHO84* promoter region due to loss of histone acetylation. In other words, *PH084* in aged *S. cerevisiae* acquires a heterochromatin epigenetic mark just like the RNAi-induced silencing mechanism characterized in *S. pombe*. In fact, the situation in *S. pombe* may be much closer to *S. cerevisiae* than would be thought at first glance. *S. pombe* uses not only the RITS complex to induce hetero-

chromatin but also an independent process involving the recruitment of histone deacetylases (HDACs). Furthermore, this latter process appears to be the dominant gene-silencing pathway used for the region of the genome involved in the mating type switch (Yamada et al., 2005). Also, dissection of the RNAi mechanism of gene silencing in *S. pombe* shows that the exosome and in particular an associated polyA polymerase (Cid14) are required for gene silencing (Buhler et al., 2007). The fact that silencing of the *PHO84* gene involves both the exosome (Rrp6) and HDACs strikingly parallels these aspects of silencing in *S. pombe*. Indeed, in using both RNAi and HDAC-associated pathways for gene silencing, *S. pombe* demonstrates its evolutionary position at center stage between *S. cerevisiae* and mammals.

As with all advances, new answers raise new questions. First, it is important to know how many other genes in *S. cerevisiae* besides *PHO84* are subject to a similar process of stress-induced gene silencing. Clearly, expression array analysis is called for here. Also, from an evolutionary perspective it is interesting that for *S. pombe* and also possibly in plants, RNAi-induced gene silencing is the predominant use of RNAi. In contrast, in higher eukaryotes, RNAi appears to primarily downregulate mRNA expression and inhibit translation efficiency in the cytoplasm via the actions of siRNAs and microRNAs. Apparently, there are no clear protein homologs in *S. cerevisiae* of the well-defined RNAi apparatus in *S. pombe* and higher eukaryotes. However, some surprises may await us that may reveal a more unifying mechanism for all eukaryotic gene silencing. Clearly, the rapid degradation of newly synthesized CUTs, both sense and antisense, is a key aspect of gene silencing in eukaryotes.

REFERENCES

Buhler, M., Haas, W., Gygi, S.P., and Moazed, D. (2007). Cell *129*, 707–721.

Camblong, J., Iglesias, N., Fickentscher, C., Dieppois, G., and Stutz, F. (2007). Cell, this issue.

Greger, I.H., Aranda, A., and Proudfoot, N. (2000). Proc. Natl. Acad. Sci. USA *97*, 8415–8420.

Grewal, S.I., and Jia, S. (2007). Nat. Rev. Genet. *8*, 35–46.

Hongay, C.F., Grisafi, P.L., Galitski, T., and Fink, G.R. (2006). Cell *127*, 735–745.

Martens, J.A., Laprade, L., and Winston, F. (2004). Nature *429*, 571–574.

Prescott, E.M., and Proudfoot, N.J. (2002). Proc. Natl. Acad. Sci. USA *99*, 8796–8801.

Shearwin, K.E., Callen, B.P., and Egan, J.B. (2005). Trends Genet. *21*, 339–345.

Wyers, F., Rougemaille, M., Badis, G., Rousselle, J.C., Dufour, M.E., Boulay, J., Regnault, B., Devaux, F., Namane, A., Seraphin, B., et al. (2005). Cell *121*, 725–737.

Yamada, T., Fischle, W., Sugiyama, T., Allis, C.D., and Grewal, S.I. (2005). Mol. Cell *20*, 173–185.

RecBCD: The Supercar of DNA Repair

Dale B. Wigley[1,*]

[1]Cancer Research UK Clare Hall Laboratories, The London Research Institute, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3LD, UK

*Correspondence: dale.wigley@cancer.org.uk

DOI 10.1016/j.cell.2007.11.004

The DNA helicase RecBCD pauses when it reaches recombination hotspots known as Chi sites and then proceeds at a slower speed of translocation than before Chi recognition. Reporting in this issue, Spies et al. (2007) now show that this reduction in translocation velocity occurs when RecBCD changes which of its two motor subunits is in the lead.

Motor car fans will be familiar with the Bugatti Veyron, until a few weeks ago the world's fastest supercar. The Veyron is an astonishing engineering achievement powered by the fusion of two V8 engines to create a single W16 quad-turbocharged motor that produces 1001 bhp and is capable of speeds of over 250 mph. Many readers of *Cell*, however, may be more familiar with the "supercar" of DNA repair, RecBCD. This enzyme is responsible for initiating repair of double-strand breaks in many bacteria. Like the Veyron, RecBCD contains two engines (the RecB and RecD helicase motor subunits; see Figure 1) that are capable of driving the complex along DNA at over 1000 base pairs per second. The RecB and RecD motors are each powered by hydrolysis of ATP, the combination consuming two ATP molecules per base pair. Significantly, RecBCD is more cleverly engineered than the Veyron because the two motors can work independently. In fact, in work presented in this issue, Spies et al. (2007) show that following the recognition of recombination hotspots called Chi (*c*rossover *h*otspot *i*nstigator) sites, RecBCD is able to switch which of its two motors takes the lead and thereby regulate the translocation velocity of the complex.

Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Microbiology

RNA interference: roles in fungal biology

Hitoshi Nakayashiki and Quoc Bao Nguyen

The discovery of RNA interference (RNAi) has been the major recent breakthrough in biology. Only a few years after its discovery, RNAi has rapidly become a powerful reverse genetic tool, especially in organisms where gene targeting is inefficient and/or time-consuming. In filamentous fungi, RNAi is not currently used as widely as is gene targeting by homologous recombination that works with practical efficiencies in most model fungal species. However, to explore gene function in filamentous fungi, RNAi has the potential to offer new, efficient tools that gene disruption methods cannot provide. In this review, possible advantages and disadvantages of RNAi for fungal biology in the postgenomics era will be discussed. In addition, we will briefly review recent discoveries on RNAi-related biological phenomena (RNA silencing) in fungi.

Addresses
Laboratory of Plant Pathology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

Corresponding author: Nakayashiki, Hitoshi (hnakaya@kobe-u.ac.jp)

Current Opinion in Microbiology 2008, 11:494–502

This review comes from a themed issue on
Eukaryotes
Edited by Nancy Keller

Available online 3rd November 2008

1369-5274/$ – see front matter

DOI 10.1016/j.mib.2008.10.001

Introduction

In 1996, the publication of the completely sequenced genome of *Saccharomyces cerevisiae* heralded the dawn of the genomics era for eukaryotes [1]. Since then, with the rapid development of sequencing technologies, relatively large numbers of fungal genomes have been decoded, because of their compact genome sizes. To date, over 80 genomes of fungi and yeasts have been made publicly available on web-based databases, and even more fungal genomes are being sequenced and annotated (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi). This unprecedented wealth of genetic information serves as a rich resource for reverse genetic approaches to understanding gene function.

An efficient reverse genetic approach is RNA interference (RNAi). RNAi is a technique in which double-stranded RNA triggers the degradation of a homologous mRNA, thereby diminishing or abolishing gene expression [2]. RNAi has proven effective in most eukaryotes, including vertebrates, plants, worms, protists, and fungi.

The story of RNAi in fungi began with a finding by Romano and Machino in 1992 [3], whereby the expression of the endogenous gene, *al-1*, which is involved in carotenoid biosynthesis, was attenuated by a transformation with homologous *al-1* sequences in the fungus *Neurospora crassa*. This phenomenon was designated as quelling. A series of remarkable studies on quelling-deficient mutants of *N. crassa* [4] has revealed the involvement of RNA-dependent RNA polymerase (RdRP) and argonaute protein in the pathway, providing molecular evidence that quelling belongs to a broad category of RNA-mediated post-transcriptional gene silencing, as typified by RNAi.

RNAi strategies in filamentous fungi

RNAi using a hairpin RNA (hpRNA)-expressing plasmid

Although 'canonical' quelling is induced by the transformation with partial coding sequence that is homologous to an endogenous target [3], more recently it has been shown that hpRNA-expressing constructs induce more efficient and stable silencing [5]. The first example of fungal RNAi by an hpRNA-expressing plasmid was demonstrated by Liu *et al.* in the basidiomycetous yeast *Cryptococcus neoformans* [6]. To date, plasmid constructs expressing hpRNA or intron-containing hpRNA (ihpRNA) are the most prevalent and reliable platforms to induce RNAi in fungi [7–10]. These types of vector have been successfully used to demonstrate RNAi using model genes and to explore gene function in a wide range of fungal species and fungus-like organisms, such as Oomycetes (water molds) [11,12] and Myxomycetes (slime molds) [13] (Table 1).

To facilitate the construction of an ihpRNA-expressing plasmid by polymerase chain reaction (PCR)-based cloning, the versatile vector pSilent-1 (available from the Fungal Genetic Stock Center (http://www.fgsc.net/)) was developed for ascomycete fungi [14]. Recently, Shafran *et al.* have introduced the Gateway technology into pSilent-1 and established the high-throughput RNAi vector, pTroya [15]. Similarly, pFANTAi4, which uses the Gateway technology and a green fluorescent protein (GFP) sentinel system, has been developed for the human pathogenic fungus *Blastomyces dermatitidis* [16••]. Those vectors are applicable for large-scale functional genomic experiments in yeasts and filamentous fungi.

RNAi using an opposing-dual promoter system

While an ihpRNA-expressing plasmid is useful for inducing RNAi in fungi, its applicability is generally limited to

Table 1

Various types of RNAi in fungi and fungus-like organisms.

Species	RNAi trigger	Transformation	Reference
Ascomycota			
Neurospora crassa	Homologous transgene	PEG-mediated method	[3][a]
Magnaporthe oryzae	IR[b]	PEG-mediated method	[7]
Histoplasma capsulatum	IR	Electroporation	[17]
Aspergillus nidulans	Synthetic siRNA	Uptake from culture medium	[21]
Basidiomycota			
Cryptococcus neoformans	IR	Electroporation	[6]
Coprinus cinereus	IR	Lithium acetate method	[8]
Schizophyllum commune	IR	PEG-mediated method	[9]
Zygomycota			
Mucor circinelloides	Homologous transgene	PEG-mediated method	[70]
Mortierella alpina	IR	Microparticle bombardment	[71]
Oomycota[c]			
Phytophthora infestans	Homologous transgene	PEG-mediated method[d]	[11]
P. infestans	Homologous transgene	Electroporation	[12]
P. infestans	dsRNA	Lipofectin-mediated transfection	[22]
Myxomycete (Slim mold)[b]			
Dictyostelium discoideum	IR	Electroporation	[13]

[a] First reports in a fungal class or induction method are listed as representatives.
[b] IR, hairpin RNA or inverted repeat RNA-expressing plasmid.
[c] Fungus-like organisms.
[d] Lipofectin was added to increase transformation efficiency.

small or moderate scale analyses since the construction of such vectors normally requires two steps of orientated cloning. One solution for this limitation of the ihpRNA-expressing plasmid is the Gateway technology, as described above. As an alternative, RNAi vectors with an opposing-dual promoter system, which enables vector construction with a single, nonorientated cloning step, have been developed for *Histoplasma capsulatum*, *Magnaporthe oryzae*, and *C. neoformans* [17,18••] (I Bose, TL Doering, unpublished). In these systems, sense and antisense RNA of the target gene, which is expected to form dsRNA in the cell, are transcribed independently under the control of the two opposing RNA polymerase II promoters (Figure 1).

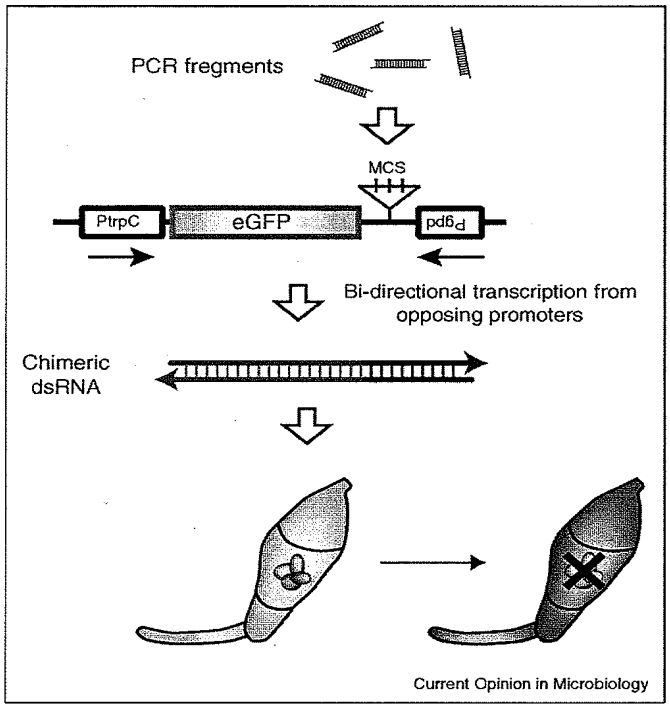
Rappleye *et al.* reported that a silencing vector of this type induced only moderate silencing of *eGFP* (35% reduction on average) in *H. capsulatum* [17]. Recently, pSilent–Dual1 (pSD1), carrying opposing *trp*C and *gpd* promoters, has been constructed and used in *M. oryzae*. Consistent with the case of *H. capsulatum*, the efficacy of gene silencing by pSD1 was generally lower than that exhibited by ihpRNA-expressing vectors [18••]. Nevertheless, strong gene silencing (>80% reduction) was induced in a small fraction of the resulting transformants. To facilitate efficient screening for strongly silenced transformants, a fragment of the *eGFP* gene was introduced into pSD1 so that a target gene is transcribed as a chimera with *eGFP* (Figure 1). In this screening system GFP fluorescence provides an effective indicator to select transformants in which interference was operating. This cosilencing-based screening has been successfully demonstrated in *C. neoformans*, *Venturia inaequalis*, *Acremonium chrysogenum*, and *B. dermatitidis* using GFP, DsRed or endogenous maker genes [6,16••,19,20]. In RNAi experiments, the screening of silenced transformants or cell lines is a time-consuming process, especially when silencing of the target gene produces no obvious phenotype. The cosilencing-based screening strategy could be one of the solutions for the labor-intensive aspect of RNAi systems.

Using the pSD1 system, a series of knockdown mutants of almost all known calcium signaling related genes in the genome of *M. oryzae* was generated and examined for phenotypic defects [18••]. This represented the first high-throughput RNAi experiments performed in filamentous fungi, providing novel evidence for the involvement of several new calcium signaling related genes in infection-related development and pathogenicity of the fungus. The phenotypic defects observed in the knockdown mutants were mostly, albeit with some exceptions, consistent with those reported in knockout and/or pharmacological experiments in yeast or other fungi. Therefore, the pSD1 and related systems may serve as a feasible alternative for exploring gene function in fungi. Despite some limitations discussed later, high-throughput RNAi methods should prove valuable, especially as a first screening tool before detailed analyses.

Direct delivery of siRNA/dsRNA into fungal cells

Even though the direct delivery of synthetic siRNA to cultured cells is a common method to introduce RNAi in

Figure 1



Screening for knockdown mutants by cosilencing with the green fluorescent protein (GFP) gene in the dual promoter systems. A target gene fragment is amplified by polymerase chain reaction (PCR) using a set of specific primers and inserted into a silencing vector with a GFP fragment. Chimeric RNAs are transcribed bidirectionally by the opposing promoters in the fungal cell expressing GFP. Using GFP fluorescence as an indicator of gene silencing, knockdown mutants of the target gene are screened and subjected to further analyses.

mammalian systems, to date, such applications have been very rarely reported in fungi. In *Aspergillus nidulans*, ornithine decarboxylase (ODC), a key polyamine biosynthesis gene, was specifically silenced by treating germinating spores with synthetic 23 nt siRNA duplex with 2 nt overhangs at the 3′-terminus [21]. The treatment of fungal spores with 5–25 nM ODC siRNA duplex in culture medium caused a significant reduction in spore germination and germ tube growth together with silencing of the ODC gene. ODC mRNA was reduced to the lowest level after 18 hours of incubation, and lasted until 48 hours of incubation. Those results indicated that siRNA duplex could be taken up by germinating fungal spores from the culture medium, providing a rapid and convenient method to induce RNAi in fungal cells. The applicability of synthetic siRNA-directed RNAi in other fungal species remains to be examined.

In addition, a method for the direct delivery of longer dsRNA into protoplasts has been reported in *Phytophthora infestans*, which belongs to the fungus-like Oomycetes [22]. A marker gene, GFP, and two *P. infestans* genes, *inf1* and *cdc14* were transiently silenced by Lipofectin-mediated transfection of protoplasts with *in vitro* synthesized Cy3-labeled dsRNAs (150–300 bp in size). In the case of the marker gene GFP, gene silencing was induced in the regenerating protoplasts for up to four days after exposure to GFP dsRNA. Thereafter, GFP fluorescence partially recovered but remained at a significantly reduced level (7–67% of the controls) for 17 days after transfection [22]. When the highly expressed gene *inf1* was targeted for RNAi, a significant reduction in *inf1* mRNA expression was usually detected only 12–15 days after transfection. In mammalian systems, synthetic siRNA-directed RNAi is typically activated within hours or one to two days and remains effective for several days after transfection. Since the original trigger dsRNA molecules may not be intact two weeks after transfection, some signal amplification mechanism is likely to be involved in the apparent late occurrence of gene silencing in *P. infestans*.

Advantages and disadvantages of RNAi as a genetic tool in fungal biology

Knockout or knockdown?

As a genetic tool, RNAi has several characteristics that contrast with those of the conventional gene disruption methods, which can make RNAi either advantageous or disadvantageous. First, RNAi causes only a partial reduction (knockdown) but not a complete loss (knockout) in gene expression. Incomplete gene suppression by RNAi is generally regarded as a drawback since it could result in phenotypic variations that sometimes make the interpretation of RNAi data difficult.

In another respect, however, incomplete gene suppression can be a characteristic of merit. For example, partial silencing by RNAi makes it possible to investigate the effects of an essential gene on a phenotype of interest. Compared with conventional approaches for the analysis of such genes (such as the use of temperature-sensitive mutants), RNAi offers a more convenient and effective tool, especially by the combination with an inducible promoter that would allow gene expression to be diminished at specific stages during development [23•].

In addition, it should be noted that knockdown technology can, in some cases, provide more detailed information compared to stable knockouts since rates of gene suppression in RNAi experiments can be variable among treated cell lines or transformants, while gene suppression in knockout experiments is fixed at 100%. For instance, by using a series of RNAi transformants showing different levels of gene suppression, it may be possible to assess how much inhibition of gene expression is actually required to cause a certain phenotypic defect. The minimum effective inhibition rate would differ among different target genes, and it could represent the degree of

impact of the gene on the phenotype. This type of information could be important, for example in drug target research.

In this regard, in *M. oryzae*, only a slight decrease in gene expression caused a complete loss of infection-related morphogenesis and pathogenicity in RNAi mutants of some calcium signaling related genes (e.g. phospholipase C, *Dun1*-like genes, and *End3*-like genes) [18••], while for other genes, such as fungal hydrophobin *Mpg1*, even strong knockdown did not severely affect the pathogenicity of the RNAi mutants [14], despite the fact that its knockout mutants showed a drastic reduction in pathogenicity. Thus, the calcium signaling related genes may have a bigger impact on the infection-related morphogenesis and pathogenicity of the fungus compared to the fungal hydrophobin.

Locus-dependent or sequence-dependent?

A second difference between RNAi and gene disruption approaches is that the former suppresses gene expression in a sequence-specific manner, while the later is locus specific. The sequence-specific characteristics of RNAi could be effectively used to analyze a functionally redundant gene family since family members sharing high sequence similarity can be simultaneously silenced by a single RNAi construct [24,25]. This approach should be significantly less time-consuming compared to constructing a multiple-gene knockout mutant. As described previously, chimeric constructs are also useful to knockdown even multiple distantly related target genes by a single transformation [6].

For fungal or fungal-like species that have multinuclear heterokaryotic mycelia, RNAi possibly offers a valuable gene analysis tool since the RNAi machinery is known to degrade cognate mRNA in the cytoplasm and, therefore, is likely to be operative against any mRNA in multinuclear heterokaryotic mycelia. Indeed, internuclear transfer of gene silencing was shown in heterokaryotic *N. crassa* strains and in the multinuclear heterokaryotic Oomycete *P. infestans* [12,26]. In addition, locus-independent RNAi is applicable to fungal species in which the efficacy of homologous recombination is below a practical level.

Furthermore, the sequence specific nature of RNAi provides new ingenious techniques of gene analysis, such as selective silencing of alternatively spliced variants [27], and the resurrection method by which an engineered protein with numerous synonymous substitutions is selectively expressed while its endogenous original protein is silenced [28]. These methods should open new possibilities for elucidating gene function in fungi.

However, as a negative aspect of sequence specific silencing by RNAi, it causes a crucial technical problem of the so-called 'off-target effects', which lead to changes in expression of unintended genes having partial complementarity of the sense or antisense strands to the target gene. Currently, off-target effects are the main concern in RNAi experiments since they can cause a false positive as well as a false negative result [29]. In human cell culture systems, microarray analyses have revealed that off-target effects include both inhibition and stimulation of non-target genes [30], and likely operate through both siRNA and miRNA pathways [31]. Compared to synthetic siRNA used in mammalian RNAi systems, the use of long dsRNA triggers has been thought to reduce the probability of off-target effects since a variety of different siRNAs should be produced by Dicer from the longer precursor, resulting in a relatively low concentration of a specific siRNA species that can induce off-target effects on a nontargeted gene. However, it has been recently shown that off-target effects mediated by short homology stretches (as short as 16 nt) within long dsRNAs are prevalent in *Drosophila* RNAi screens [32•].

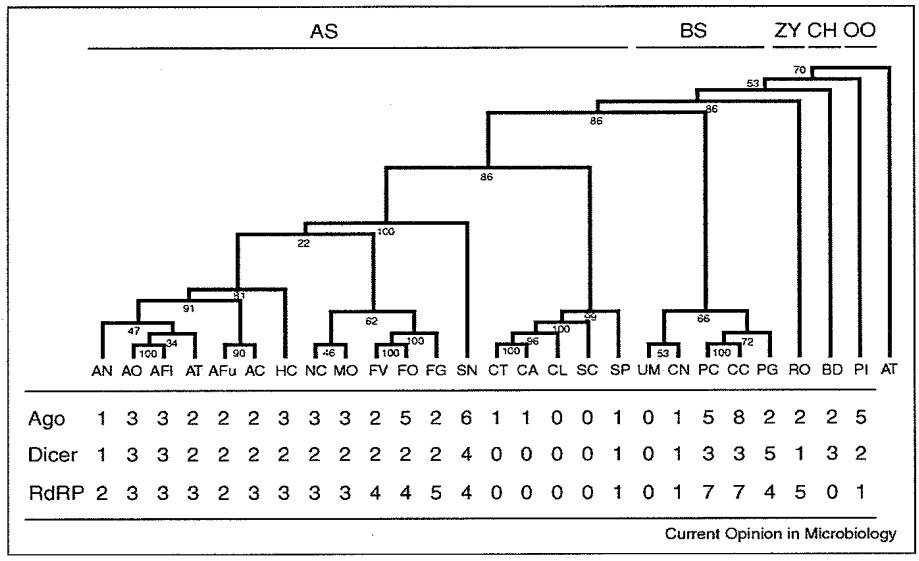
In *M. oryzae*, the extent of off-target effects was examined in the pSD1 system with genes sharing significant sequence homology (cutoff E-value at 1×10^{-3}) to the target genes [18••]. In 20 of 29 cases, the homologous nontargeted genes were simultaneously silenced at a similar level to the 'true target'. In most of the cases, as expected, the homologous nontargeted genes were other members of a multiple gene family of the target gene but a few more distantly related genes were also unexpectedly silenced. In addition, even genes that showed relatively low homology (E-value, 10^{-3} to 10^{-4}) to the target were significantly silenced in almost half of the cases. This could simply result from a downstream effect but not from 'direct' off-target effects. However, it is also possible that off-target effects in *M. oryzae* may involve genes with very low sequence homology. To manage off-target effects in fungal RNAi systems, it would be important to carefully examine a minimum sequence similarity that can cause cosilencing in a fungal species.

Another disadvantage of RNAi, arising from its sequence specific nature, is that it is technically difficult to verify the RNAi results by genetic complementation. In gene disruption experiments, genetic complementation by a functional gene gives unambiguous evidence to identify the causal gene. Such a simple verification method is so far not available in RNAi experiments. A resurrection gene with synonymous substitutions can be useful for this purpose, but it would be practically challenging to apply it to a large-scale experiment.

Biological and physiological roles of RNA silencing pathways in fungi

Recent evidence indicates that noncoding small RNAs play critical roles in a greater variety of cellular processes

Figure 2



Phylogenetic relationship of the fungal species based on beta-tubulin sequences and the numbers of Argonaute (Ago)-, Dicer-, and RNA-dependent RNA polymerase (RdRP)-like proteins predicted in the genome sequences. The amino acid alignment of the fungal beta-tubulin genes was generated by Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) with the default parameter settings. The distance tree was constructed by the Neighbour-Joining method and their robustness was estimated by performing 1000 bootstrap replicates. Arabidopsis was used as an outgroup to root the tree. The numbers of the RNA silencing proteins were first estimated by performing BLAST searches with conserved motifs of Argonaute, Dicer, and RdRP proteins against the public databases with the default parameter settings. For further selection, flanking sequences of the candidates were examined for typical features of the RNA silencing proteins as described previously [33]. AS, ascomycete; BS, basidiomycetes; ZY, zygomycete; OO, oomycete; AC, *Aspergillus clavatus*; AFl, *Aspergillus flavus*; AFu, *Aspergillus fumigatus*; AN, *Aspergillus nidulans*; AO, *Aspergillus oryzae*; AN, *Aspergillus terreus*; CA, *Candida albicans*; CC, *Coprinus cinereus*; CL, *Candida lusitaniae*; CN, *Cryptococcus neoformans*; CT, *Candida tropicalis*; FG, *Fusarium graminearum*; FO, *Fusarium oxysporum*; FV, *Fusarium verticillioides*; HC, *Histoplasma capsulatum*; MO, *Magnaporthe oryzae*; NC, *Neurospora crassa*; PC, *Phanerochaete chrysosporium*; PG, *Puccinia graminis*; PI, *Phytophthora infestans*; RO, *Rhizopus oryzae*; SC, *Saccharomyces cerevisiae*; SN, *Stagonospora nodorum*; SP, *Schizosaccharomyces pombe*; UM, *Ustilago maydis*; AT, *Arabidopsis thaliana*.

than was once assumed. The proposed biological roles of small RNA-mediated gene silencing pathways (referred to hereafter as RNA silencing pathways) in eukaryotes include regulation of endogenous gene expression, resistance to viruses, transposon silencing, heterochromatin formation, programmed DNA elimination, and maintenance of genome stability in reproductive cells. The RNA silencing pathways seem to have diversified during the evolution of eukaryotes, and therefore some of the pathways are likely to be absent in certain eukaryotes, or present in only a limited class of organisms.

Even within the kingdom fungi, RNA silencing pathways appear to have diversified significantly because the numbers of RNA silencing proteins differ considerably among fungal species [33] (Figure 2). However, relatively little is known about the physiological roles of the fungal RNA silencing pathways. In some fungal species, such as *S. cerevisiae* and *Ustilago maydis*, the entire RNA silencing machinery appears to be lost [34,35], indicating that RNA silencing pathways may be dispensable for fundamental metabolism and development in fungi. Interestingly, in *Ustilago hordei*, a close relative to *U. maydis*, RNA silencing has been demonstrated [36]. Therefore, the loss of the RNA silencing machinery seems to sporadically occur in the fungi kingdom, as previously shown in the protist *Trypanosomes* [37]. In support of this, *A. nidulans* appears to be 'losing' one of the two copies of dicer and argonaute genes [38•] while, intriguingly, its close relatives *Aspergillus oryzae* and *Aspergillus flavus* have likely gained extra copy of dicer and argonaute genes, possibly by gene duplication (Figure 2).

The best-studied and probably the most conserved RNA silencing pathway in fungi is the one thus far described in this review; here we call it, 'the RNAi pathway', which operates in vegetative (somatic) cells and uses siRNAs as specificity determinants. The RNAi pathway is known to be involved in transposon silencing in higher eukaryotes, and that is also likely to be the case in fungi [39,40]. Recently, a series of studies from Donald Nuss laboratory has clearly shown that this pathway functions as an antiviral defense mechanism in the chestnut blight fungus *Cryphonectria parasitica* [41,42•,43]. Similarly, mycovirus defense via the RNAi pathway was demonstrated in *A. nidulans* [44]. The expression of the RNAi component

genes was upregulated by long dsRNA and upon viral infection in *N. crassa* and *C. parasitica* [43,45••]. Interestingly, Choudhary *et al.* showed that homologs of antiviral and interferon-stimulated genes were also activated by dsRNA in *N. crassa* [45••]. Together, the RNAi pathway seems to be an ancient natural defense mechanism against viral and transposon infections that likely originated at a very early stage of eukaryotes' evolution.

Another major RNA silencing pathway known in higher eukaryotes is the miRNA pathway, in which miRNAs regulate the expression of endogenous mRNA post-transcriptionally via translational repression or mRNA degradation. miRNAs are transcribed from regions of the genome as long primary-precursors, which are subsequently processed into the mature miRNAs, usually ranging from 19 to 24 nt in length. In higher eukaryotes, such as animals and plants, miRNAs have been implicated in a variety of cellular processes including metabolism, apoptosis, differentiation, development, and response to infection. In lower eukaryotes, the occurrence of miRNAs in a unicellular organism was recently reported for the single-cell algae, *Chlamydomonas reinhardtii* [46,47]. The 'miRNAs' in *Chlamydomonas* likely caused target mRNA cleavage rather than translation inhibition as reported with plant miRNAs. In addition, miRNA-like small RNAs were found and shown to be developmentally regulated in the unicellular slime mold *Dictyostelium discoideum* [48•]. Biogenesis of at least one of the miRNA-like small RNAs in *D. discoideum* was dicer-dependent [48•].

In contrast, to our knowledge, no miRNA has so far been identified in fungi. Notably, however, developmental defects were observed in some cases of null mutants of fungal dicer proteins. In *N. crassa*, homozygosity for *dcl-1* mutation was completely barren [49] while dicer disruptants in *M. oryzae* and *Mucor circinelloides* appeared to have slight morphological abnormalities in vegetative mycelia [50,51]. Even though the molecular mechanisms leading to the developmental defects in the fungi are unknown, the fungal dicers appear to have a role in more than defense against viruses and transposable elements.

In the fission yeast *Schizosaccharomyces pombe*, RNA silencing components are known to be involved in sequence-specific heterochromatin formation via histone modifications [52]. RNA silencing dependent transcriptional gene silencing through sequence-specific DNA methylation, as well as histone modifications, was also observed in plants [53]. Analogous pathways are likely to operate in *Tetrahymena*, *Drosophila melanogaster*, *Caenorhabditis elegans* and possibly in mammals [54]. However, in the fungus *N. crassa*, RNA silencing components were shown to be dispensable for either *de novo* DNA methylation or histone modifications [55,56]. In *M. oryzae*, *de novo* DNA methylation was also independent of siRNA production (unpublished data). Therefore, RNA silencing dependent transcriptional gene silencing may be broadly but not universally conserved in eukaryotes.

Meiotic silencing by unpaired DNA (MSUD) identified in *N. crassa* is a unique RNA silencing pathway that operates for a limited period during the sexual phase from an early stage of meiosis after karyogamy until ascospore maturation [57,58]. MSUD silences the expression of genes that exist in one parental chromosome but not in its pairing partner, thus causing unpaired DNA during meiosis. It appears that *Neurospora* has two separate RNAi-related pathways in two different phases of its life cycle; quelling for the vegetative phase and MSUD for the sexual phase. Meiotic silencing by unsynapsed regions has also been observed in *C. elegans* [59] and mouse [60,61] at the transcriptional level and possibly also at the post-transcriptional level. Therefore, the evolutionary origin of MSUD-like pathways may be very ancient.

Recently, a novel group of germ cell specific small RNAs were discovered in *Drosophila* [62•,63], zebrafish [64], and mammals [65,66]. The new class of small RNAs was designated as piRNAs, and is characterized by a distinctly larger size (24–31 nt) and by association with the PIWI subfamily of argonaute proteins. Therefore, distinct RNA silencing pathways seem to operate during vegetative and sexual phases in higher eukaryotes, as in *Neurospora*, even though the evolutionary relationship between MSUD and piRNA pathways remains to be elucidated.

Concluding remarks

RNAi is a potentially powerful tool for a wide variety of gene silencing applications. As described, the RNAi and gene disruption methods differ in principle, and therefore, have their own strengths and limitations. From the point of view of accuracy, gene destruction methods generally give better data than RNAi. The drawbacks of RNAi, such as incomplete repression and possible unintended targets, are often described for pharmacological studies. In this regard, RNAi seems to be somewhere between gene disruption and pharmacological approaches. However, the potential of RNAi is not limited to a convenient, although restricted, alternative to gene disruption. RNAi should prove useful for the studies of functional genomics in fungi in that it can provide novel and rapid gene analysis applications that gene disruption methods cannot offer. To make RNAi a better tool for gene function analysis in fungi, the next challenges are to know the extent of off-target effects in fungal cells and to develop an inducible RNAi system with a combination of a strictly controlled promoter and a convenient inducer applicable to a wide range of filamentous fungi.

In several fungal species such as *Coprinus cinereus*, *Fusarium oxysporum*, *Phanerochaete chrysosporium*, and *Stagonospora nodorum*, quite large numbers of RNA silencing proteins are predicted from genomic sequences (Figure 2). This implies that there could be 'hidden' RNA silencing pathways in fungi. The recent identification of novel RNA silencing related components such as QIP, SAD-2, and Replication Protein A (RPA) in *Neurospora* [67•,68•,69•] has demonstrated that filamentous fungi still provide an excellent model for molecular studies of RNA silencing. RNAi will play important roles in fungal biology, and, in turn, the filamentous fungus will be a powerful tool for dissecting the molecular mechanisms of RNA silencing.

Acknowledgements

I am grateful to Donald L Nuss, University of Maryland Biotechnology Institute, and Ulrich Kück, Ruhr-Universität, for the time in reviewing the manuscript and providing comments for improvement.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M *et al.*: **Life with 6000 genes.** *Science* 1996, **274**:546-567.

2. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.** *Nature* 1998, **391**:806-811.

3. Romano N, Macino G: **Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences.** *Mol Microbiol* 1992, **6**:3343-3353.

4. Cogoni C, Macino G: **Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*.** *Proc Natl Acad Sci U S A* 1997, **94**:10233-10238.

5. Goldoni M, Azzalin G, Macino G, Cogoni C: **Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*.** *Fungal Genet Biol* 2004, **41**:1016-1024.

6. Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL: **RNA interference in the pathogenic fungus *Cryptococcus neoformans*.** *Genetics* 2002, **160**:463-470.

7. Kadotani N, Nakayashiki H, Tosa Y, Mayama S: **RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*.** *Mol Plant Microbe Interact* 2003, **16**:769-776.

8. Namekawa SH, Iwabata K, Sugawara H, Hamada FN, Koshiyama A, Chiku H, Kamada T, Sakaguchi K: **Knockdown of LIM15/DMC1 in the mushroom *Coprinus cinereus* by double-stranded RNA-mediated gene silencing.** *Microbiology* 2005, **151**:3669-3678.

9. de Jong JF, Deelstra HJ, Wosten HA, Lugones LG: **RNA-mediated gene silencing in monokaryons and dikaryons of *Schizophyllum commune*.** *Appl Environ Microbiol* 2006, **72**:1267-1269.

10. Takeno S, Sakuradani E, Murata S, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S: **Establishment of an overall transformation system for an oil-producing filamentous fungus, *Mortierella alpina* 1S-4.** *Appl Microbiol Biotechnol* 2004, **65**:419-425.

11. van West P, Kamoun S, van't Klooster JW, Govers F: **Internuclear gene silencing in *Phytophthora infestans*.** *Mol Cell* 1999, **3**:339-348.

12. Latijnhouwers M, Ligterink W, Vleeshouwers VGAA, West Pv, Govers F: **A Galpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*.** *Mol Microbiol* 2004, **51**:925-936.

13. Martens H, Novotny J, Oberstrass J, Steck TL, Postlethwait P, Nellen W: **RNAi in *Dictyostelium*: the role of RNA-directed RNA polymerases and double-stranded RNase.** *Mol Biol Cell* 2002, **13**:445-453.

14. Nakayashiki H, Hanada S, Nguyen BQ, Kadotani N, Tosa Y, Mayama S: **RNA silencing as a tool for exploring gene function in ascomycete fungi.** *Fungal Genet Biol* 2005, **42**:275-283.

15. Shafran H, Miyara I, Eshed R, Prusky D, Sherman A: **Development of new tools for studying gene function in fungi based on the Gateway system.** *Fungal Genet Biol* 2008, **45**:1147-1154.

16. •• Krajaejun T, Gauthier GM, Rappleye CA, Sullivan TD, Klein BS: **Development and application of a green fluorescent protein sentinel system for identification of RNA interference in *Blastomyces dermatitidis* illuminates the role of septin in morphogenesis and sporulation.** *Eukaryot Cell* 2007, **6**:1299-1309.
The authors developed the RNAi vector, pFANTAi4 that uses the Gateway technology and a GFP sentinel system. To test the feasibility of the vector, two model genes, *BAD1* and *LacZ*, together with a previously unstudied gene encoding septin were targeted for RNAi in the human pathogenic fungus *B. dermatitidis*. The results indicated that pFANTAi4 offers a new high-throughput gene analysis tool for fungi.

17. Rappleye CA, Engle JT, Goldman WE: **RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence.** *Mol Microbiol* 2004, **53**:153-165.

18. •• Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S, Nakayashiki H: **Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system.** *Mol Microbiol* 2008, **68**:1348-1365.
In this paper, 37 calcium-signaling proteins in the phytopathogenic fungus *M. oryzae* were studied using the RNAi vector pSD1 with a convergent dual Pol II promoter system. This work represents the first systematic analysis of a whole biological signaling pathway by RNAi in fungi.

19. Fitzgerald A, Van Kan JA, Plummer KM: **Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats.** *Fungal Genet. Biol.* 2004, **41**:963-971.

20. Janus D, Hoff B, Hofmann E, Kück U: **An efficient fungal RNA-silencing system using the DsRed reporter gene.** *Appl Environ Microbiol* 2007, **73**:962-970.

21. Khatri M, Rajam MV: **Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene.** *Med Mycol* 2007, **45**:211-220.

22. Whisson SC, Avrova AO, West PV, Jones JT: **A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*.** *Mol Plant Pathol* 2005, **6**:153-163.

23. • Bromley M, Gordon C, Rovira-Graells N, Oliver J: **The *Aspergillus fumigatus* cellobiohydrolase B (cbhB) promoter is tightly regulated and can be exploited for controlled protein expression and RNAi.** *FEMS Microbiol Lett* 2006, **264**:246-254.
The *Aspergillus fumigatus* cbhB promoter was activated by carboxymethylcellulose but suppressed by glucose. An RNAi construct driven by the cbhB promoter induced a loss-of-function phenotype only in the presence of carboxymethylcellulose.

24. Zhao W, Fanning ML, Lane T: **Efficient RNAi-based gene family knockdown via set cover optimization.** *Artif Intell Med* 2005, **35**:61-73.

25. Kaur J, Sebastian J, Siddiqi I: **The Arabidopsis-mei2-like genes play a role in meiosis and vegetative growth in *Arabidopsis*.** *Plant Cell* 2006, **18**:545-559.

26. Cogoni C, Irelan JT, Schumacher M, Schmidhauser TJ, Selker EU, Macino G: **Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA–DNA interactions or DNA methylation.** *EMBO J* 1996, **15**:3153-3163.

27. Celotto AM, Graveley BR: **Exon-specific RNAi: a tool for dissecting the functional relevance of alternative splicing.** *RNA* 2002, **8**:718-724.

28. Mori M, Dohi K: **The "resurrection method" for modification of specific proteins in higher plants.** *FEBS Lett* 2005, **579**:6210-6216.

29. Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, Chanda S, Downward J, Ellenberg J, Fraser AG, Hacohen N *et al.*: **Minimizing the risk of reporting false positives in large-scale RNAi screens.** *Nat Methods* 2006, **3**:777-779.

30. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS: **Expression profiling reveals off-target gene regulation by RNAi.** *Nat Biotechnol* 2003, **21**:635-637.

31. Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS: **Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity.** *RNA* 2006, **12**:1179-1187.

32. • Ma Y, Creanga A, Lum L, Beachy PA: **Prevalence of off-target effects in *Drosophila* RNA interference screens.** *Nature* 2006, **443**:359-363.

The authors have performed a genome-wide RNAi screen in *Drosophila*. Many of the top candidates, however, exert their effects through off-target effects on known pathway components or through promiscuous off-target effects produced by tandem trinucleotide repeats present in many dsRNAs and genes.

33. Nakayashiki H, Kadotani N, Mayama S: **Evolution and diversification of RNA silencing proteins in fungi.** *J Mol Evol* 2006, **63**:127-135.

34. Aravind L, Watanabe H, Lipman DJ, Koonin EV: **Lineage-specific loss and divergence of functionally linked genes in eukaryotes.** *Proc Natl Acad Sci U S A* 2000, **97**:11319-11324.

35. Kämper J, Kahmann R, Bölker M, Ma LJ, Brefort T, Saville BJ, Banuett F, Kronstad JW, Gold SE, Müller O *et al.*: **Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*.** *Nature* 2006, **444**:97-101.

36. Laurie JD, Linning R, Bakkeren G: **Hallmarks of RNA silencing are found in the smut fungus *Ustilago hordei* but not in its close relative *Ustilago maydis*.** *Curr Genet* 2008, **53**:49-58.

37. Ullu E, Tschudi C, Chakraborty T: **RNA interference in protozoan parasites.** *Cell Microbiol* 2004, **6**:509-519.

38. • Hammond TM, Bok JW, Andrewski MD, Reyes-Domínguez Y, Scazzocchio C, Keller NP: **RNA silencing gene truncation in the filamentous fungus *Aspergillus nidulans*.** *Eukaryot Cell* 2008, **7**:339-349.

The genus *Aspergillus* seems to have experienced a variety of RNA silencing gene changes through the diversification of species.

39. Nolan T, Braccini L, Azzalin G, De Toni A, Macino G, Cogoni C: **The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in *Neurospora crassa*.** *Nucleic Acids Res* 2005, **33**:1564-1573.

40. Murata T, Kadotani N, Yamaguchi M, Tosa Y, Mayama S, Nakayashiki H: **siRNA-dependent and -independent post-transcriptional cosuppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*.** *Nucleic Acids Res* 2007, **35**:5987-5994.

41. Segers GC, van Wezel R, Zhang X, Hong Y, Nuss DL: **Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system.** *Eukaryot Cell* 2006, **5**:896-904.

42. • Segers GC, Zhang X, Deng F, Sun Q, Nuss DL: **Evidence that RNA silencing functions as an antiviral defense mechanism in fungi.** *Proc Natl Acad Sci U S A* 2007, **104**:12902-12906.

The role of RNA silencing as an antiviral defense mechanism in fungi was examined by testing the effect of dicer gene disruptions on mycovirus infection of the chestnut blight fungus *C. parasitica*.

43. Zhang X, Segers GC, Sun Q, Deng F, Nuss DL: **Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway.** *J Virol* 2008, **82**:2613-2619.

44. Hammond TM, Andrewski MD, Roossinck MJ, Keller NP: ***Aspergillus* mycoviruses are targets and suppressors of RNA silencing.** *Eukaryot Cell* 2008, **7**:350-357.

45. •• Choudhary S, Lee HC, Maiti M, He Q, Cheng P, Liu Q, Liu Y: **A double-stranded-RNA response program important for RNA interference efficiency.** *Mol Cell Biol* 2007, **27**:3995-4005.

The authors showed that dsRNA, but not short interfering RNA (siRNA), induces the expression of RNAi genes such as Argonaute and Dicer in *N. crassa*. Through a genome-wide screening, the authors identified a number of dsRNA-activated genes including additional RNAi components and homologs of antiviral and interferon-stimulated genes in the fungus. This implies that RNAi is part of an ancient innate defense response against viral and transposon infections.

46. Zhao T, Li G, Mi S, Li S, Hannon GJ, Wang XJ, Qi Y: **A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*.** *Genes Dev* 2007, **21**:1190-1203.

47. Molnar A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC: **miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*.** *Nature* 2007, **447**:1126-1129.

48. • Hinas A, Reimegård J, Wagner EG, Nellen W, Ambros VR, Söderbom F: **The small RNA repertoire of *Dictyostelium discoideum* and its regulation by components of the RNAi pathway.** *Nucleic Acids Res* 2007, **35**:6714-6726.

The authors reported the cloning and characterization of 18–26 nt RNAs in the cellular slime mold *D. discoideum*. The survey uncovered developmentally regulated miRNA candidates whose biogenesis was dependent on a Dicer homolog, DrnB.

49. Alexander WG, Raju NB, Xiao H, Hammond TM, Perdue TD, Metzenberg RL, Pukkila PJ, Shiu PK: **DCL-1 colocalizes with other components of the MSUD machinery and is required for silencing.** *Fungal Genet Biol* 2008, **45**:719-727.

50. Nicolas FE, de Haro JP, Torres-Martinez S, Ruiz-Vazquez RM: **Mutants defective in a *Mucor circinelloides* dicer-like gene are not compromised in siRNA silencing but display developmental defects.** *Fungal Genet Biol* 2006, **44**:504-516.

51. Kadotani N, Nakayashiki H, Tosa Y, Mayama S: **One of the two dicer-like proteins in the filamentous fungi *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related siRNA accumulation.** *J Biol Chem* 2004, **279**:44467-44474.

52. Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D: **RNAi-mediated targeting of heterochromatin by the RITS complex.** *Science* 2004, **303**:672-676.

53. Zaratiegui M, Irvine DV, Martienssen RA: **Noncoding RNAs and gene silencing.** *Cell* 2007, **128**:763-776.

54. Buhler M, Moazed D: **Transcription and RNAi in heterochromatic gene silencing.** *Nat Struct Mol Biol* 2007, **14**:1041-1048.

55. Freitag M, Lee DW, Kothe GO, Pratt RJ, Aramayo R, Selker EU: **DNA methylation is independent of RNA interference in *Neurospora*.** *Science* 2004, **304**:1939.

56. Chicas A, Forrest EC, Sepich S, Cogoni C, Macino G: **Small interfering RNAs that trigger posttranscriptional gene silencing are not required for the histone H3 Lys9 methylation necessary for transgenic tandem repeat stabilization in *Neurospora crassa*.** *Mol Cell Biol* 2005, **25**:3793-3801.

57. Aramayo R, Metzenberg RL: **Meiotic transvection in fungi.** *Cell* 1996, **86**:103-113.

58. Shiu PKT, Raju NB, Zickler D, Metzenberg RL: **Meiotic silencing by unpaired DNA.** *Cell* 2001, **107**:905-916.

59. Maine EM, Hauth J, Ratliff T, Vought VE, She X, Kelly WG: **EGO-1, a putative RNA-dependent RNA polymerase, is required for heterochromatin assembly on unpaired DNA during *C. elegans* meiosis.** *Curr Biol* 2005, **15**:1972-1978.

60. Turner JM, Mahadevaiah SK, Fernandez-Capetillo O, Nussenzweig A, Xu X, Deng CX, Burgoyne PS: **Silencing of**

unsynapsed meiotic chromosomes in the mouse. *Nat Genet* 2005, **37**:41-47.

61. Baarends WM, Wassenaar E, van der Laan R, Hoogerbrugge J, Sleddens-Linkels E, Hoeijmakers JH, de Boer P, Grootegoed JA: **Silencing of unpaired chromatin and histone H2A ubiquitination in mammalian meiosis**. *Mol Cell Biol* 2005, **25**:1041-1053.

62. • Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD: **A distinct small RNA pathway silences selfish genetic elements in the germline**. *Science* 2006, **313**:320-324.
A novel RNA silencing pathway specific to germline cells was identified in *Drosophila*. The new class of small RNA was a little longer than siRNAs in somatic cells, and associated with the Piwi Argonaute protein subfamily.

63. Saito K, Nishida KM, Mori T, Kawamura Y, Miyoshi K, Nagami T, Siomi H, Siomi MC: **Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome**. *Genes Dev* 2006, **20**:2214-2222.

64. Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB *et al.*: **A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish**. *Cell* 2007, **129**:69-82.

65. Girard A, Sachidanandam R, Hannon GJ, Carmell MA: **A germline-specific class of small RNAs binds mammalian Piwi proteins**. *Nature* 2006, **442**:199-202.

66. Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE: **Characterization of the piRNA complex from rat testes**. *Science* 2006, **313**:363-367.

67. • Maiti M, Lee HC, Liu Y: **QIP, a putative exonuclease, interacts with the *Neurospora* Argonaute protein and facilitates conversion of duplex siRNA into single strands**. *Genes Dev* 2007, **21**:590-600.
A novel RNA silencing component was identified in *Neurospora* through biochemical purification of the QDE-2 agronaute protein. A QDE-2 interactor, designated as QIP is a putative exonuclease that facilitates conversion of duplex siRNA into single strands.

68. • Shiu PK, Zickler D, Raju NB, Ruprich-Robert G, Metzenberg RL: **SAD-2 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1 RNA-directed RNA polymerase**. *Proc Natl Acad Sci U S A* 2006, **103**:2243-2248.
By screening of random insertion mutants, a novel component in the MSUD pathway was isolated and designated as SAD-2. SAD-2 colocalized with SAD-1 RdRP protein in the perinuclear region.

69. • Nolan T, Cecere G, Mancone C, Alonzi T, Tripodi M, Catalanotto C, Cogoni C: **The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in *Neurospora crassa* interacts with replication protein A**. *Nucleic Acids Res* 2008, **36**:532-538.
The authors showed physical interaction between QDE-1 RdRP and RPA, part of the DNA replication machinery. In addition, by a pharmacological approach, the authors demonstrated that the accumulation of short interfering RNAs was dependent on an ongoing DNA synthesis. Those results imply a potential link between RNA silencing and DNA replication.

70. Nicolas FE, Torres-Martinez S, Ruiz-Vazquez RM: **Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes**. *EMBO J.* 2003, **22**:3983-3991.

71. Takeno S, Sakuradani E, Tomi A, Inohara-Ochiai M, Kawashima H, Ashikari T, Shimizu S: **Improvement of the fatty acid composition of an oil-producing filamentous fungus, Mortierella alpina 1S-4, through RNA interference with delta12-desaturase gene expression**. *Appl. Environ. Microbiol.* 2005, **71**:5124-5128.